Supplemental Methods

Production, Expression, and Quantification of FV Recombinants

A pMT2 vector containing the full-length cDNA of human FV (pMT2-FV) was used as a template for creating the mutant recombinant expression vectors. A full-length recombinant of the wild-type protein with the A2440G (GenBank M16967) mutation that would result in a serine to glycine change at amino acid 756 (FV-S756G) was created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The splice variant mutant vector (FV-702 or rFV-Short) was created by cloning in an insert with A2440G and a deletion of base pairs 2441 to 4546 that correlate to the in-frame deletion of amino acids 756 through 1458. To make the insert, the full-length vector was used as a template to create two PCR products with primer-generated homologous overlapping ends to the left and right of the splice junction. The left PCR used 5'-GA TGG CAT CTG ACC AAG GT**C** AAT ATT ACT TGG GGA AGA ATA ATT TGA ACC AAC-3' and 5'-GCC AGC CGC CCC TAT AGC ATT TAC C-3', while the right PCR product used the following primers: 5'-CT TCC CCA AGT AAT ATT **G**AC CTT GGT CAG ATG CCA TCT CC-3' and 5'-GTA GGC CCA AGC CCG ACA GGC AG-3'. The PCR reactions contained 1X PCR buffer, 0.2 mM each dNTP, 150ng pMT2-FV, 50 pmols of each primer, and 2.5mU Pfu DNA polymerase (Stratagene) into a 50µL total reaction volume. The PCR reactions initiated at 94°C for 2 minutes, amplified for 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 62°C for 2 minutes, and elongated for 10 minutes at 62°C. Purified PCR products were used as templates for generating an overlapping PCR product. For this, equal amounts of purified PCR right and left products were combined with 1X PCR buffer, 0.2 mM each dNTP, 50 pmols of each of the outer flanking primers, and 2.5 mU Pfu DNA polymerase into a 50µL total reaction volume and subjected to the aforementioned PCR conditions. This final 1770 bp product was subjected to SnaB1 and Bsu36I restriction enzyme (New England Biolabs, Ipswich, MA) digestion overnight at 37°C per manufacturer's instructions and then ligated using T4 DNA ligase (Takara Bio Inc, Shiga, Japan) into purified SnaB1/Bsu36I digested pMT2FV. Vectors were sequenced to verify mutations.

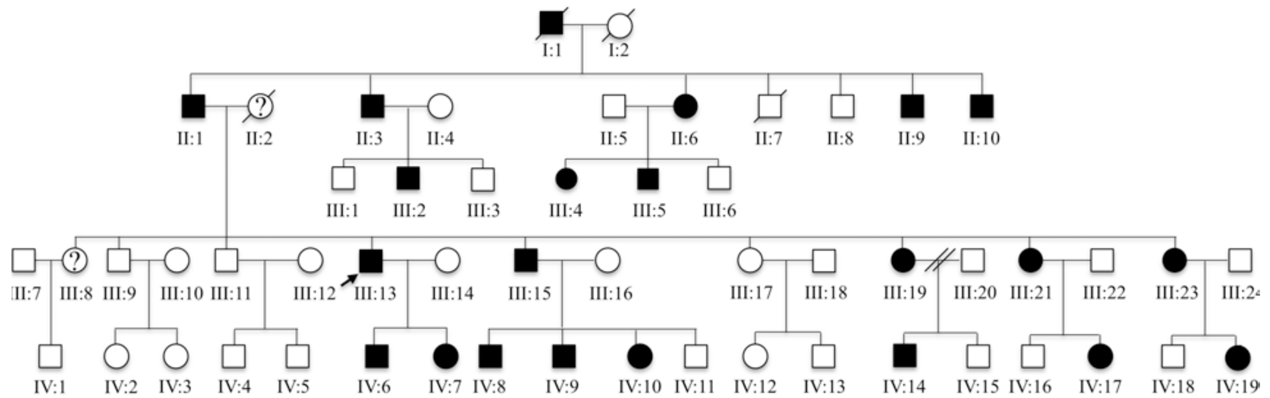
Recombinant wild-type and mutant vectors were transiently transfected into COS1 cells using the diethyl aminoethyl (DEAE)-dextran method essentially as described previously (1-3). Briefly, each recombinant vector was incubated with cells for 4 hours in Tris (pH 7.3), 0.1 mM

chloroquine, and DEAE dextran in Dulbecco's modified essential medium (Gibco, Paisley, Scotland). Cells were shocked with 10% dimethyl sulfoxide for 2 minutes and allowed to generate protein for 60-70 hours. Recombinant proteins were harvested in serum-free medium (Optimem Glutamax, Gibco) and concentrated using 100,000 MWCO Vivaspin (GE Healthcare). Aliquots were stored at -80ºC. Concentrations of the recombinant proteins were determined by a PTase assay and an ELISA as previously described(4). The expression levels of Wt FV and FV-Short were found to be similar at approximately 0.8-1 nM. The ratio between activity assessed by a PTase assay and antigen levels determined by a FV ELISA using a combination of polyclonal anti-FV (#8806 a rabbit antibody made in the laboratory) and a light chain FV monoclonal antibody (HV1, Sigma-Aldrich, Saint-Louis, Missouri) were found to be similar for the two recombinant variants (data not shown). Activation of rFV-WT and rFV-Short was monitored via immunoblot over varying concentrations of thrombin (0-2 U/mL) and normal production of the heavy and light chains were found (data not shown).

1. Kaufman, R.J. 1990. Vectors used for expression in mammalian cells. *Methods Enzymol* 185:487.511.
2. Pittman, D.D., Tomkinson, K.N., and Kaufman, R.J. 1994. Post-translational requirements for functional factor V and factor VIII secretion in mammalian cells. *J Biol Chem* 269:17329-17337.
3. Pittman, D.D., Tomkinson, K.N., Michnick, D., Selighsohn, U., and Kaufman, R.J. 1994. Posttranslational sulfation of factor V is required for efficient thrombin cleavage and activation and for full procoagulant activity. *Biochemistry* 33:6952-6959.
4. Norstrom, E., Thorelli, E., and Dahlback, B. 2002. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood* 100:524-530.
5. Swart, A.C., Klaassen, B.H., Bloys-van, T.-d., and Hemker, H.C. 1972. The adsorption of blood coagulation factors II, VII, IX and X from human plasma to aluminium hydroxide. *Thromb Diath Haemorrh* 27:490-501.

Supplemental figures with legends

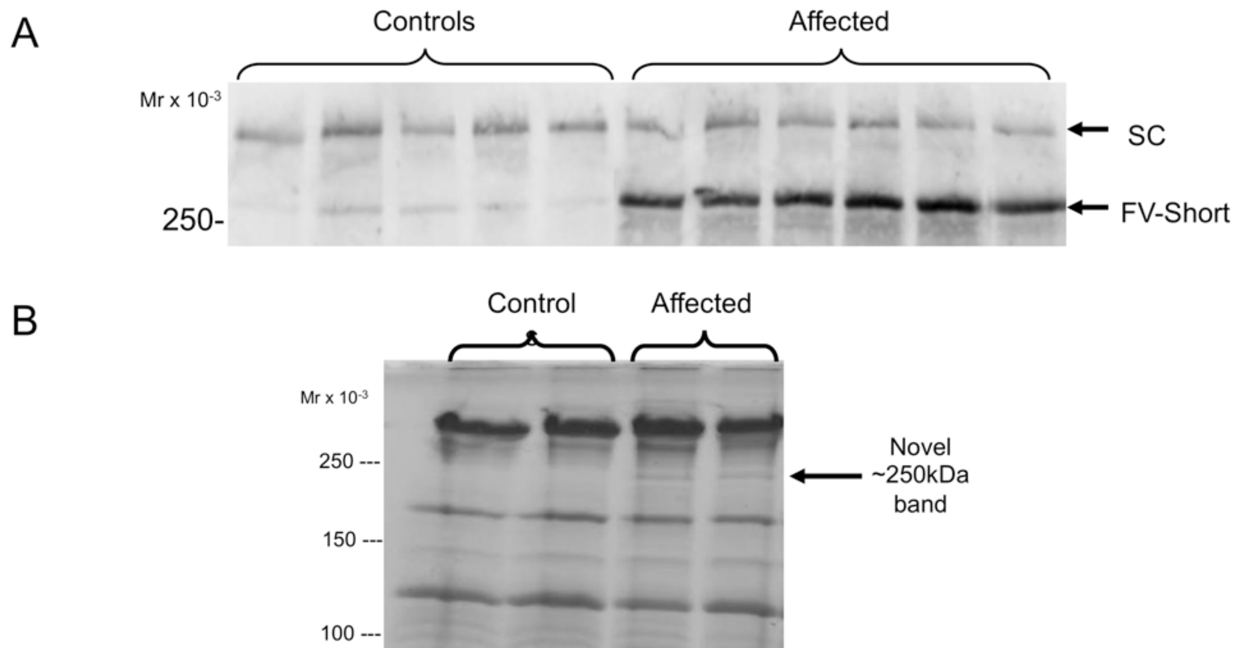
Suppl Fig. 1



Supplemental Figure 1. Pedigree of family with the East Texas bleeding disorder. Closed symbols, affected individuals with A2440G; open symbols, unaffected family members; ?, disease status unknown. Arrow point at the index case of the family.

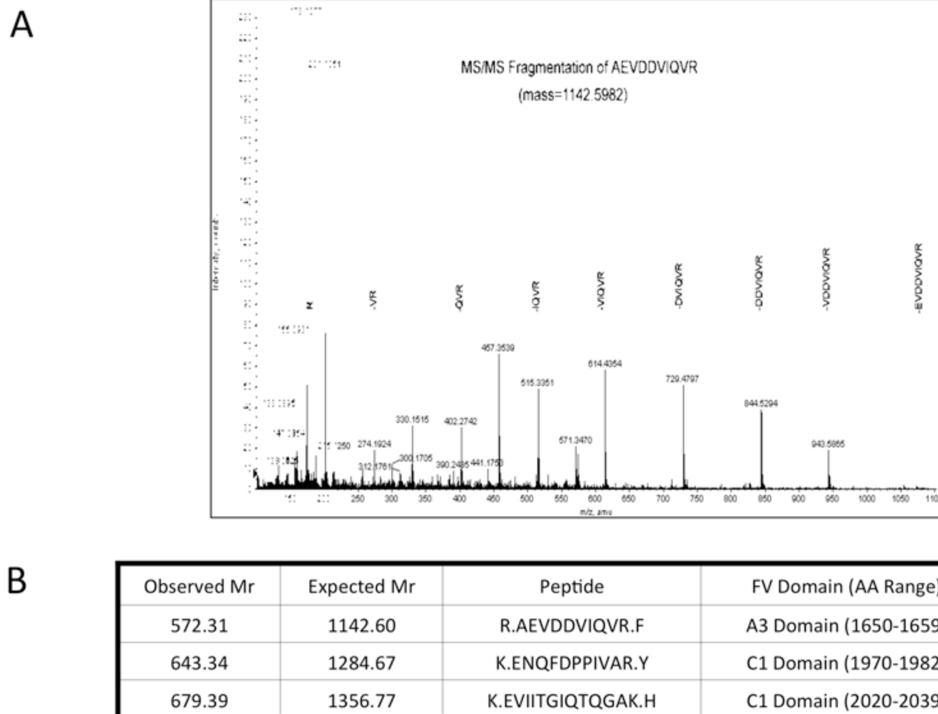
Suppl Fig. 2

Supplemental Figure 2. Conservation of A2240 and Ser756. Multiple alignments and evolutionary conservation of 46 vertebrate species generated by Multiz and other tools found within the UCSC genome web browser located at http://genome.ucsc.edu. (A) Nucleotide alignments surrounding A2440. (B) Protein alignments surrounding S756.



Supplemental Figure 3. Partial purification of FV-Short protein band from affected patient plasma. This procedure was modified from Swart et al(5). Nine parts of citrated whole plasma, protected with 1X Complete™ EDTA (ethylene diamine tetraacetic acid)-free Protease inhibitor (Roche, Indianapolis, IN), was mixed with one part aluminum hydroxide suspension (Fisher Scientific, Houston, TX). After adsorption, the sediment was sequentially washed in $^{1}/_{10}$ of the original plasma volume in 0.3M Na_2EDTA pH 8.0 (VKW1), 0.1M sodium citrate pH 8.0 (VKW2), and 0.25M potassium phosphate pH 8.0 (VKE) to elute bound proteins. FV-Short was found to be eluted in VKW2. This figure contains the 10% SDS-PAGE of VKW2 of controls ((A) Lanes 1-5, (B) Lanes 1-2) and affected patients ((A) Lanes 6-11, (B) Lanes 3-4). (A) Immunoblotting with AHV-5146 and (B) Coomassie staining. FV was detected using AHV-5146 against the heavy chain and the Amersham ECF Western Blotting Detection Kit was used to develop the western blots. Mr indicates molecular range; SC, wild-type FV single chain; and FV-Short, novel FV Short band Mr indicates molecular range.

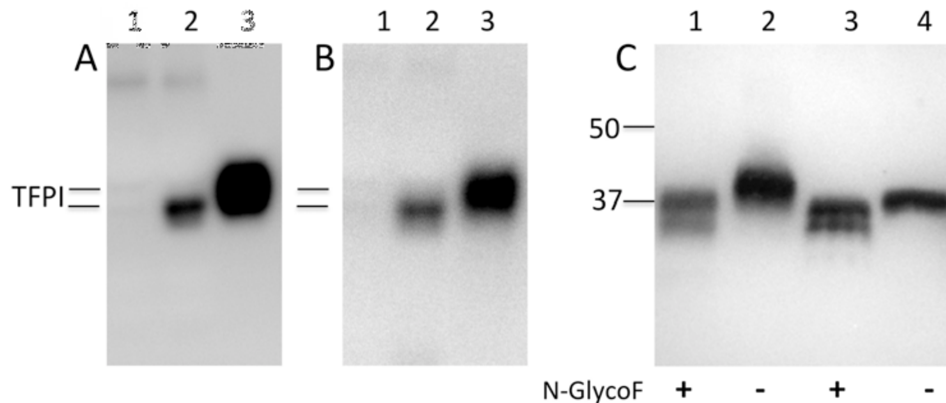
A



B

Observed Mr	Expected Mr	Peptide	FV Domain (AA Range)
572.31	1142.60	R.AEVDDVIQVR.F	A3 Domain (1650-1659)
643.34	1284.67	K.ENQFDPPIVAR.Y	C1 Domain (1970-1982)
679.39	1356.77	K.EVIITGIQTQGAK.H	C1 Domain (2020-2039)

Supplemental Figure 4. HPLC/MS-MS analysis of FV-Short derived from affected patient plasma. (A) An example of HPLC-TOF MS/MS chromatogram of a peptide from ~250kDa protein band identified as FV-Short. (B) Peptides found from MS/MS analysis that indicate that FV-Short contains multiple light chain residues of factor V. HPLC-TOF MS/MS and peptide analysis completed by the University of Texas Health Science Center at Houston Proteomic Core Facility. (Mr, molecular range; AA, amino acid).



Supplemental Figure 5. Comparison between plasma-derived and recombinant TFPIα: effects of deglycosylation. A and B, pooled plasma (≈1 ul) from unaffected (lanes 1) and affected (lanes 2) individuals and rTFPIα (lanes 3 ≈1 ng/lane) were analyzed by immunoblotting using either TFPIα monoclonal antibodies against the N-terminus (A) (AHTPFI-5138) or C-terminus (B) (Epitomics). C, the eluate from an anti-TFPI immune precipitation of affected plasma (as described in Figure 10) and rTFPIα were treated with N-Glycosidase F (Roche) following the manufacturer's instructions. The samples were then analyzed with immuno blotting using the TFPIα monoclonal antibody AHTFPI-5138. Lanes 1 and 2, treated and untreated rTFPIα, respectively. Lanes 3 and 4, treated and untreated TFPI-immune precipitate, respectively.